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Nrf2-Keap-1 imbalance under acute shear stress induces inflammatory response in venous endothelial cells

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Abstract:	Vascular endothelial cells stimulation is associated with the activation of different signalling pathways and transcription factors. Acute shear stress is known to induce different pro-inflammatory mediators such as IL-8. Nrf2 is activated by prolonged high shear stress promoting an antiinflammatory and athero-protective environment. However, little is known about the impact of acute shear stress on Nrf2 and Keap1 function and its role in IL-8 regulation. We aimed to examine Nrf2-Keap1 complex activation in-vitro and its role in regulating IL-8 transcripts under acute arterial shear stress (12dyn/cm2) in venous endothelial cells (ECs). We note that acute high shear stress caused a significant upregulation of Nrf2 target genes, HO-1 and GCLM and an increased IL-8 upregulation at 90 and 120 min. Mechanistically, acute high shear did not affect Nrf2 nuclear translocation but resulted in reduced nuclear Keap1, suggesting that the reduction in nuclear Keap1 may result in increased free nuclear nrf2 to induce transcription. Consistently, the suppression of Keap1 using shRNA (shKeap1) resulted in significant upregulation of IL-8 transcripts in response to acute shear stress. Interestingly; the over expression of Nrf2 using Nrf2-Ad-WT or Sulforaphane was also associated with significant upregulation of IL-8 compared to controls. This study highlights the role of Keap1 in Nrf2 activation under shear stress and indicates that activation of Nrf2 may be deleterious in ECs in the context of acute haemodynamic injury.

Abstract:

Vascular endothelial cells stimulation is associated with the activation of different signalling pathways and transcription factors. Acute shear stress is known to induce different pro-inflammatory mediators such as IL-8. Nrf2 is activated by prolonged high shear stress promoting an antiinflammatory and athero-protective environment. However, little is known about the impact of acute shear stress on Nrf2 and Keap1 function and its role in IL-8 regulation. We aimed to examine Nrf2-Keap1 complex activation in-vitro and its role in regulating IL-8 transcripts under acute arterial shear stress (12dyn/cm^2) in venous endothelial cells (ECs). We note that acute high shear stress caused a significant upregulation of Nrf2 target genes, HO-1 and GCLM and an increased IL-8 upregulation at 90 and 120 min. Mechanistically, acute high shear did not affect Nrf2 nuclear translocation but resulted in reduced nuclear Keap1, suggesting that the reduction in nuclear Keap1 may result in increased free nuclear nrf2 to induce transcription. Consistently, the suppression of Keap1 using shRNA (shKeap1) resulted in significant upregulation of IL-8 transcripts in response to acute shear stress. Interestingly; the over expression of Nrf2 using Nrf2-Ad-WT or Sulforaphane was also associated with significant upregulation of IL-8 compared to controls. This study highlights the role of Keap1 in Nrf2 activation under shear stress and indicates that activation of Nrf2 may be deleterious in ECs in the context of acute haemodynamic injury.

Introduction

Regulation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is achieved through physical interaction with its innate repressor, Kelch-like ECH-associating protein 1 (Keap1), which is required for both cytosolic sequestration and continuous targeted degradation of the transcription factor (1-3). Activation of the Keap1-Nrf2 complex is achieved via stress-sensing mechanisms, both intrinsically, within the complex itself, and extrinsically, through stress-responsive Mitogen activated protein kinase (MAPK) controlled phosphorylation (4, 5). Upon activation, Keap1 undergoes a conformational change and dissociates from Nrf2. This allows the transcription factor to bind to its reciprocal antioxidant response element (ARE) and regulate myriad stress-response target genes, including phase II detoxifying and antioxidant enzymes and glutathione producers such as heme-oxygenase 1 (HO-1) and glutamylcysteine ligase modifier (GCLM) (1, 3). Prolonged high shear stress at athero-protected arterial sites is known to promote a quiescent, antiinflammatory and antioxidant status in endothelial cells (ECs) partly due to Nrf2 activation (5, 6).

It is recognised that veins in the human body are exposed to low shear stress compared with arteries. The acute stimulation of venous ECs by sudden haemodynamic changes such as grafting of a long saphenous vein into the arterial circulation can be associated with the upregulation of different inflammatory mediators and signalling pathways such as MAPK and NF-kB (7, 8). However, little is known about Nrf2-Keap1 responses to an acute onset of shear stress in these venous ECs.

Interleukin-8 (IL-8) is a chemoattractant cytokine produced by ECs and activates neutrophils at inflammatory sites. IL-8 is regulated by MAPK, NF-kB and Nrf2 (9, 10) . Furthermore, it has been demonstrated that IL-8 can be activated by acute high shear stress or prolonged low shear (7, 11).

In this study, we investigate the impact of acute shear stress in human umbilical vein ECs (HUVECs) on Nrf2-Keap1 complex and its potential role in mediating IL-8 transcription response.

2. Materials and Methods

EC culture and shear stress

1mm thick Menzel glass microscope slides (Thermo Scientific, USA) were strerilised and air-dried, a physical barrier area to cell growth was established by adhering modified, reusable flexiPERM cell culture chambers (Sarstedt, Germany) to the glass slide surface. The established growth area was coated with 700µl 1%(w/v) gelatin (Sigma Aldrich, USA) for 1 hour at 37°C; the gelatin solution was then aspirated, prior to washing the slide with Dulbecco's PBS and subsequent addition of 3mL pre-warmed Ready-to-use Endothelial Cell Growth Medium (ECGM: Promocell, Germany), containing final concentrations of 0.004 ml/ml endothelial cell growth supplement, 0.1 ng/ml recombinant human epidermal growth factor, 1ng/ml recombinant human basic fibroblast growth factor, 90µg/ml heparin, 1µg/ml hydrocortisone, 100µg/ml penicillin, 100IU/ml streptomycin and 2mM L-Glutamine (Promocell and Gibco, USA). Human Umbilical Vein Endothelial Cells (HUVECs) (pooled from up to 4 donors) were purchased from Promocell (Germany). $2x10^5$ cells/slide were cultured (≤Passage 4) for 48 hours to full confluency on glass slides (checked visually under microscope), with ECGM. HUVECs were then cultured overnight in Endothelial Cell Basal Medium (ECBM: Promocell) supplemented with 2% (v/v) FCS, 100µg/mL penicillin, 100IU/mL streptomycin and 2µmol/L L-Glutamine (with supplements removed).

For shear stress exposure, HUVECs were exposed to laminar, unidirectional acute (≤4 hours) high shear stress at 12 dyn/cm^2 to simulate arterial rates of shear stress, for varying time points using parallel plate flow chambers (Supplementary figure 1) or maintained in static conditions.

Briefly, the glass slides were placed into the parallel plate chambers and sealed with a silicon sheet gasket. A reservoir containing 30mL M199 GlutaMax culture medium (Gibco, USA), supplemented with 2% (v/v) FCS, 100µg/ml penicillin and 100U/ml streptomycin, was attached to a closed-circuit loop of silicon tubing (VWR, USA and Elkay, UK) and connected to the chambers. HUVECs were then cultured at 37°C and 5% CO2 and shear stress was applied using a multi-channel peristaltic pump (Watson-Marlow, UK) in a dedicated incubator to maintain the temperature. Shear stress rates were calculated for a slit die assuming the viscosity of water at 37°C, using the following equation: $\tau=6\mu Q/wh^2$. Where τ represents Shear Stress; μ, viscosity; Q, flow rate; w, width and h, height.

Pharmacological over expression of Nrf2

Pharmacological activation and over expression of Nrf2 was performed prior to shear stress exposure using Sulforaphane (SFN), a naturally occurring isothiocyanate with Nrf2-inducing and antiinflammatory functions (5, 12). HUVECs were either pre-treated with SFN (Sigma Aldrich, USA) dissolved in DMSO or DMSO only for 4 hours. A final concentration of 5µmol/mL was used as it was associated with maximum nuclear Nrf2 concentration (Supplementary figure 2A).

Adenoviral-mediated expression of WT and DN-Nrf2

Overexpression of wild-type Nrf2 (WT-Nrf2) and dominant-negative Nrf2 (DN-Nrf2) was achieved using recombinant adenoviruses (provided by Professor Paul Evans, University of Sheffield). The WT is known to result in over expression of Nrf2 while the DN results in the overexpression of a form of the protein which lacks the N-terminal transcriptional activation domain, but the C-terminal DNA-binding and heterodimerisation domains are retained, thus, competes with endogenous Nrf2 for binding to the ARE-motif without affecting the Keap1 (13). The empty cassette (rAd66) was used as the negative control. Firstly, HUVECs were

seeded in 6 well plates for assessment of overexpression or onto 1% gelatin-coated glass slides and cultured for 24 hours, as above, for shear stress exposure. The rAd66, DN-Nrf2 and WT-Nrf2 containing adenoviruses were diluted in ECBM to achieve a final concentration of $1x10^7$ pfu/mL. Cells were cultured with adenoviruses for 18 hours and, after washing cells with DPBS, fresh ECBM added for a further 48 hours to enable maximal expression of viral proteins. Expression of DN-Nrf2 and WT-Nrf2 was assessed by Western blotting (WB) (Supplementary figure 2 B, C).

Adenoviral-mediated knockdown of Keap1

Short hairpin RNAs against Keap1 (shKeap1) were cloned into pCpG-free MCS vectors (Invivogen) and the expression cassette was inserted into pDC511 (Microbix Biosystems, Canada) for adenoviral vector production, described previously (14). Knockdown of Keap1 was performed similarly to above; briefly, HUVECs were cultured for 24 hours in ECGM, then incubated for a further 18 hours with $1x10^7$pfu/mL Ad-shKeap1, or Ad-shLuciferase as a vehicle control, in ECBM. Following removal of the virus, cells were cultured for 24 hours and either analysed for Keap1 knockdown or exposed to shear stress.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

mRNA transcript levels were quantified by RT-qPCR with gene-specific primers for pro-inflammatory genes: Interleukin-6 (IL-6), Interleukin-8 (IL-8); and ARE/Nrf2 target genes: Glutamate-cysteine ligase, modifier sub-unit (GCLM), and HO-1. Extraction and purification of total RNA was performed using miRNeasy Mini kit (Qiagen, Germany), as per manufacturer's instructions. Reverse transcription of total RNA was performed using transcriptor first strand cDNA synthesis kit (Roche, Switzerland). All RT-qPCR reactions were performed in triplicate. Expression of the specific target genes relative to the house-keeping gene, β-Tubulin, were calculated using the delta-delta CT method (15).

Immunoblotting

Total HUVEC protein was extracted with 1% (w/v) SDS lysis buffer, or, alternatively, cell compartment lysates were prepared with the NE-PER Nuclear and Cytoplasmic extraction kit (ThermoFisher). All Polyacrylamide Gel Electrophoresis (PAGE) were performed using the Bio-Rad Mini format 1-D electrophoresis system and 4-15% Mini-PROTEAN TGX stain free gels (Bio-Rad, USA). Gels were photoactivated using the ChemiDoc XRS+ imaging system (Bio-Rad) for endpoint normalisation of Western blot data to total protein concentration per lane. Following transfer, 0.2µM Nitrocellulose membranes (Bio-Rad), were blocked in 5% (w/v) semi-skimmed milk powder and primary antibodies were incubated overnight at 4°C (See supplementary methods for details). Primary antibodies were detected by Horseradish Peroxidase-conjugated secondary antibodies for 1 hour at room temperature and Luminata Forte Western HRP substrate reagent (Millipore, USA). Membranes were imaged using the ChemiDoc XRS+ imaging system and detected protein bands were quantified by densitometry and normalised to the stain-free loading control.

Primary antibodies used were as follows: Nrf2, rabbit anti-Nrf2(c20) (0.2µg/mL; Santa Cruz Biotechnology, sc-722); Keap1, goat anti-Keap1 (0.4µg/mL; Santa Cruz Biotechnology, sc-15246); Lamin A/C, rabbit anti-Lamin A/C (0.33µg/mL; Santa Cruz Biotechnology, sc-6215) for 1 hour at RT; Glyceraldehye 3-Phosphate dehydrogenase (GAPDH), mouse anti-GAPDH (3.33µg/mL; Millipore, MAB374).

Statistical analysis

For experiments where only two groups were analysed, data were subjected to a paired, two-tailed t-test. For experiments where more than two groups were analysed, a one- or two-way ANOVA was used depending on the number of independent variables, followed by post-hoc pairwise comparisons with Bonferroni correction for multiple comparisons. The cut-off value

for statistical significance was 0.05. Data are presented as mean±SEM of 4-6 independent experiments. All statistical analysis was performed with GraphPad Prism 7.0.

Results

1- Nrf2 and Keap 1 activation under acute shear stress

HUVECs exposure to acute shear stress at 12dyn/cm^2 for 30 minutes did not result in changes in nuclear Nrf2 suggesting that acute shear stress is not associated with Nrf2 activation (nuclear translocation) at that time point (figure 1). Interestingly, we noted significant reduction in nuclear Keap1 with a concomitant increase in cytosolic Keap 1 suggesting that acute shear stress can result in Keap1 export from the nucleus (figure 1). These changes were associated with increased expression of Nrf2 regulated genes (HO-1 and GCLM) (figure 2 A, B) and an increase in IL-8 transcript levels after 90 and 120 minutes of exposure to acute shear stress compared to static controls (figure 2 C). These findings indicate that although acute shear stress did not increase Nrf2 nuclear translocation however, the reduction in nuclear Keap1 may have resulted in the presence of more unbound (active) Nrf2 in the nucleus to activate HO-1 and GCLM transcription, which occurs at the same time as the upregulation of pro-inflammatory IL-8.

The findings above raised the possibility that Keap1 reduction and associated increased levels of free Nrf2 can tip the fine balance of the tightly controlled Nrf2-Keap1 system, leading to increased levels of unbound Nrf2, which may contribute to an EC inflammatory response under acute shear stress conditions.

To test the above hypothesis, we reasoned that the reduction of Keap1 may increase inflammatory responses to acute shear stress. Using a targeted shRNA (shKeap1), Keap 1 was knocked down by 25% as demonstrated by reduced Keap-1 transcripts and increased HO-1 transcripts (figure 2 D, E) The knock down of Keap1 resulted in increase in IL-8 transcripts at

static and under 90 and 120 minutes of exposure to acute shear stress compared to shLuc (figure 2 F).

2- Hyperactivation of Nrf2 and acute shear stress leads to induction of a pro-inflammatory gene response

To further examine the role of Keap1-Nrf2 imbalance under acute shear stress, we investigated the role of over-activation of Nrf2 on responses to acute shear stress.

Nrf2 over-activation was performed pharmacologically, using SFN, a naturally occurring isothiocyanate with Nrf2-inducing and antiinflammatory functions (5, 12) or via an adenoviral-mediated overexpression of WT-Nrf2. SFN treatment of HUVECs increased mRNA levels of HO-1 under static conditions and after exposure to acute shear stress at 90 and 120 minutes compared to DMSO control (Figure 3A). We noted significant increase in IL-8 transcripts in SFN-treated samples in static and acute shear stress-exposed HUVECs compared to DMSO control (figure 3B).

To confirm the above results, we transfected HUVECs with WT- Nrf2 $1x10^7$pfu/mL which resulted in a significant increase in Nrf2 protein levels was associated with an increase in HO-1 transcripts at static and under acute shear stress compared to control (figure 3C). Again we noted a concomitant upregulation of IL-8 at static and under acute shear stress (figure 3D).

To determine if the IL-8 increase is Nrf2 dependent, we inhibited Nrf2 using an adenoviral-mediated overexpression of a dominant-negative form of the protein (DN-Nrf2) which lacks the N-terminal transcriptional activation domain, but the C-terminal DNA-binding and heterodimerisation domains are retained, thus, competes with endogenous Nrf2 for binding to the ARE-motif (13). Following treatment with DN-Nrf2, HUVECs were exposed to shear stress for 90 and 120 minutes. Acute shear stress did not result in HO-1 activation compared to control under static or acute shear stress conditions as expected (figure 3E), but, more

importantly, we observed no suppression in IL-8 response to acute shear stress compared to rAd66 at the same time points of exposure to shear (figure 3F).

Discussion

Inflammation and oxidative stress are inextricably linked to vascular injury associated with damage to the vascular wall or harmful haemodynamic stimulation (5, 7, 8). Such pathogenic shifts in the endothelium, from quiescence to dysfunction, results in a change in gene expression profiles from antiinflammatory and antioxidant, to proinflammatory and oxidative (7, 8, 16-18). Here, we investigated the response of the Nrf2-Keap1 complex to acute shear stress stimulation in venous ECs to simulate exposure of venous ECs to arterial shear stress levels during venous grafting and examined the role that Nrf2 activation has on ARE and IL-8 pro-inflammatory gene expression.

We noted that HO-1 and GCLM transcript levels were increased under acute shear stress indicative of the ARE-gene response activation. However, similarly to what we previously reported, acute shear stress was not associated with increased nuclear translocation of Nrf2 in venous ECs (8). When we looked at Keap1 expression and translocation, we noted a reduction in nuclear Keap1, suggesting that Keap1 reduction in the nucleus may allow for more ARE activation due to the presence of more unbound Nrf2 in the nucleus.

It has been previously observed that shear stress exposure improves Nrf2 stability via lipid peroxidation by NADPH oxidase (NOX) action, which can oxidatively modify Keap1 to induce its dissociation from Nrf2 (19). Additionally, covalent modification of Nrf2, by PKC-induced phosphorylation at Serine residue 40, is recognised to further stabilise Nrf2 and further supress its association with Keap1, which may possibly explain the increased ARE mRNA response (6, 20, 21), but cannot explain the reduction in Keap1 under acute shear conditions.

As previously reported, acute shear stress was associated with the activation of pro-inflammatory IL-8 transcripts (7); however, surprisingly we noted that inhibiting Keap1 increased IL-8 upregulation in response to acute shear stress compared to controls, despite increased activation of the protective ARE gene response basally. Moreover, the over expression of Nrf2 was also associated with increased pro inflammatory response to acute shear stress demonstrated by increased transcript levels of IL-8. Furthermore, the presence of DN-Nrf2, which is not known to influence Keap1, did not suppress IL-8, despite suppressing HO-1 activation.

It has been demonstrated previously that the exposure of arterial ECs in-vitro and in-vivo to prolonged high shear stress is associated with increased nuclear Nrf2 and the presence of antiinflammatory antiatherosclerotic environment (5, 22). However, it seems that in-vitro hyperactivation of Nrf2 can impair EC adhesion and repair (23). Moreover, global deletion of Nrf2 ($Nrf2^{-/-}$) in hypercholesterolemic mice was associated with reduced atherosclerosis, despite the predicted reduction in antioxidant defence and inflammation (24, 25). While this has been explained previously by effects on lipid metabolism (24), our results suggests that Nrf2-Keap1 pathway is more complicated and Keap1 may play a more important role in regulating the positive or negative effects of Nrf2 in different contexts.

Recent evidence from cancer studies are beginning to reveal the possible negative impacts that the activation of Nrf2 can have in pathological conditions. This evidence points towards the unconstrained activity of Nrf2 in cancer as deleterious and emphasises the importance of context and dose dependency with Nrf2 activation (26-28). Furthermore, Taguchi et al, examined the effects of graded reduction of Keap1 expression in adult mice. When challenged with APAP, $Keap1^{flox/-}$ mice were more protected from mortality than wild-type and even $Keap1^{flox/-}$::Albumin-Cre mice. In contrast, a decrease in Keap1 levels to less than 50%

resulted in increased mortality in a study of 2-year-old mice indicating that the sustained systemic activation of Nrf2 is disadvantageous (29).

Our current study highlights two new findings. Firstly, we demonstrated for the first time that acute high shear stress can lead to increased Keap1 export from the nucleus. Secondly that a nuclear reduction in Keap1 can lead to more free nuclear Nrf2, resulting in ARE gene activation, but, more importantly, seems to contribute to the pro-inflammatory response following acute high shear stress by regulating IL-8 transcripts.

Our study has a few limitations. It is an in-vitro study that aimed mainly to look at the activity of Nrf2 under acute shear stress. The use of shKeap1 only resulted in 25% reduction of Keap1, however, this seems to be enough to increase ARE regulated gene HO-1. Furthermore, it is important to point that this experimental model used culture media not blood as the circulating fluids and the temperature was maintained during the experiment under rigid control which is different to the circulating fluids in the human body and the fluctuation of temperature that can affect viscosity (30). Moreover, this current model does not take into account any potential turbulent flow patterns generated at the anastomosis site in non-interposition grafts models (31, 32). It will be interesting in the future to investigate in depth the impact of Keap1 overexpression on inflammatory responses and to validate these findings in-vivo or ex-vivo where such limitation can be minimised.

Conclusion

The findings presented here, regarding the paradoxical, pro-inflammatory function of Nrf2 under acute shear stress, represent a contrasting view from the widely accepted notion that Nrf2 possesses antiinflammatory potential, highlighting the importance of context and levels with this transcription factor. These observations indicate the necessity for tight regulation of the

Nrf2-dependent transcriptional programme and raise a cautionary note on modulation of Nrf2 as an adjunctive therapy to reduce inflammation.

Disclosure: none of the authors have conflict of interest to declare.

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Figure legends

Figure 1. Nrf2 and Keap 1 activation under acute shear stress

Representative image of WB of cytosolic and nuclear Nrf2 and Keap1 from HUVECs exposed to acute shear stress for 30 mins or kept under static conditions, all WBs were normalised using bands detected on stain free gel and expressed as a fold change in protein level relative to static. Approximate molecular weights in kDa are indicated adjacent to representative blots (n=6).

Figure 2. ARE genes and IL-8 activation under acute shear stress

(A) Relative mRNA levels of HO-1, (B) GCLM and (C) IL-8 from HUVECs exposed to acute shear stress for 90 and 120 minutes or maintained in static conditions (n=4). (D) Relative mRNA levels of Keap1 and (E) HO-1 in HUVECs infected with adenoviruses containing shRNAs against Keap1 (shKeap1), or adenoviral vector control containing shRNAs against Renilla Luciferase (shLuc), at $1x10^7$pfu/mL (n=4). (F) Relative mRNA levels of IL-8 in and subsequently exposed to acute shear stress for 90 and 120 minutes or maintained in static conditions (n=4). mRNA levels were normalised to β-tubulin and expressed as a fold-change relative to static control.

Figure 3. Hyperactivation of Nrf2 and acute shear stress leads to induction of a proinflammatory gene response

Relative mRNA levels of HO-1(3A) and IL-8 9 (3B) in HUVECs pre-treated with 5μmol/L SFN, or vehicle, DMSO (0.05% (v/v)), and subsequently exposed to acute shear stress for 90 and 120 minutes or maintained in static conditions. Relative mRNA levels of HO-1(3C) and IL-8 (3D) from HUVECs infected with WT-Nrf2 containing- or rAd66 empty vector adenovirus at $1x10^7$ pfu/mL and subsequently exposed to acute shear stress for 90 and 120 minutes or maintained in static conditions. Relative mRNA levels of HO-1(3E) and IL-8 (3F) from HUVECs infected with DN-Nrf2 containing- or rAd66 control-adenoviruses, at $1x10^7$ pfu/mL, and subsequently exposed to acute shear stress for 90 and 120 minutes or maintained in static conditions. mRNA expression data were normalised to β-tubulin and expressed as a fold-change relative to rAd66 static control (n=4).

Figure. 1

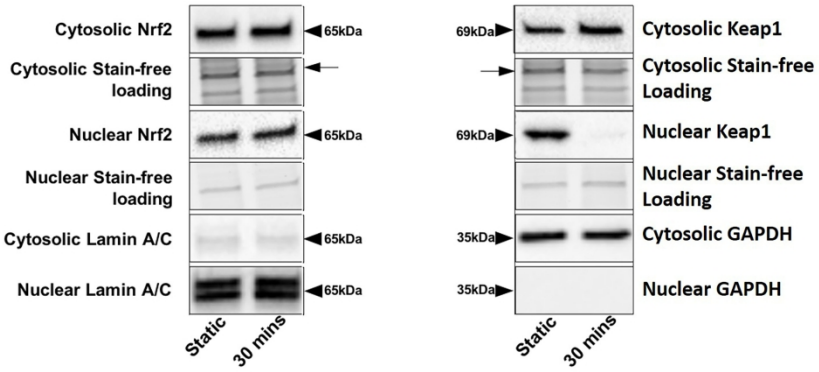

Figure 1. Nrf2 and Keap 1 activation under acute shear stress

294x132mm (300 x 300 DPI)

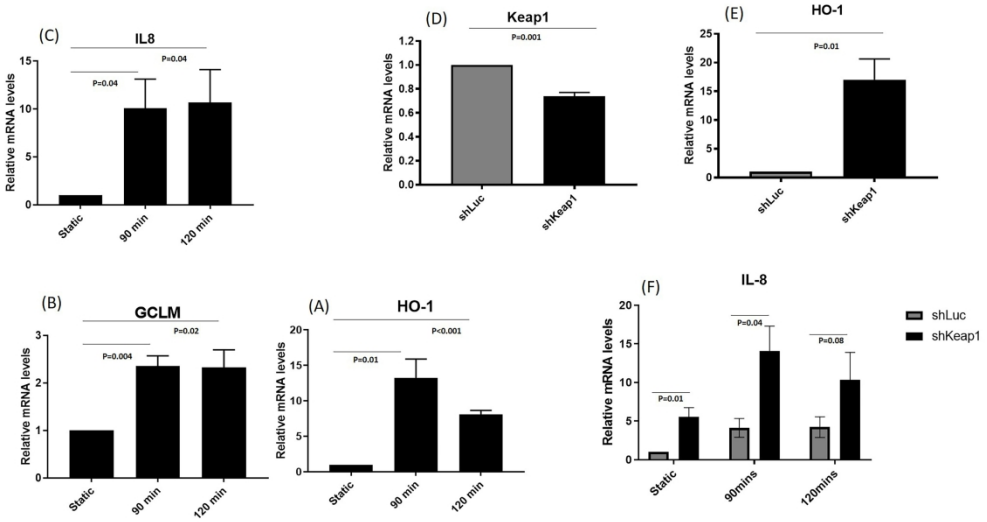

Figure 2. ARE genes and IL-8 activation under acute shear stress

351x190mm (300 x 300 DPI)

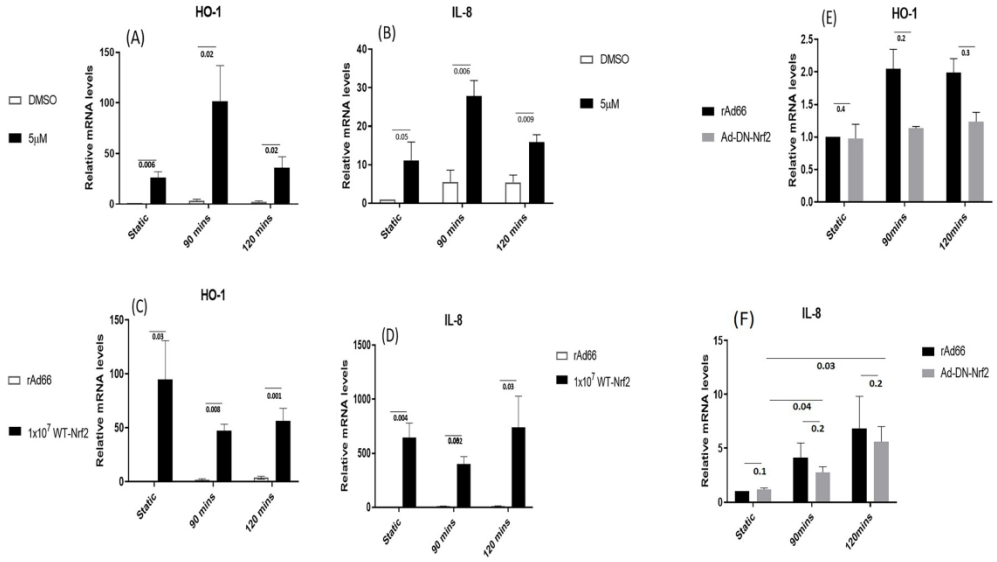

Figure 3. Hyperactivation of Nrf2 and acute shear stress leads to induction of a proinflammatory gene response

308x179mm (300 x 300 DPI)

Nrf2-Keap-1 imbalance under acute shear stress induces inflammatory response in venous endothelial cells

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Running Title: *Nrf2 hyperactivation induces inflammation in ECs*

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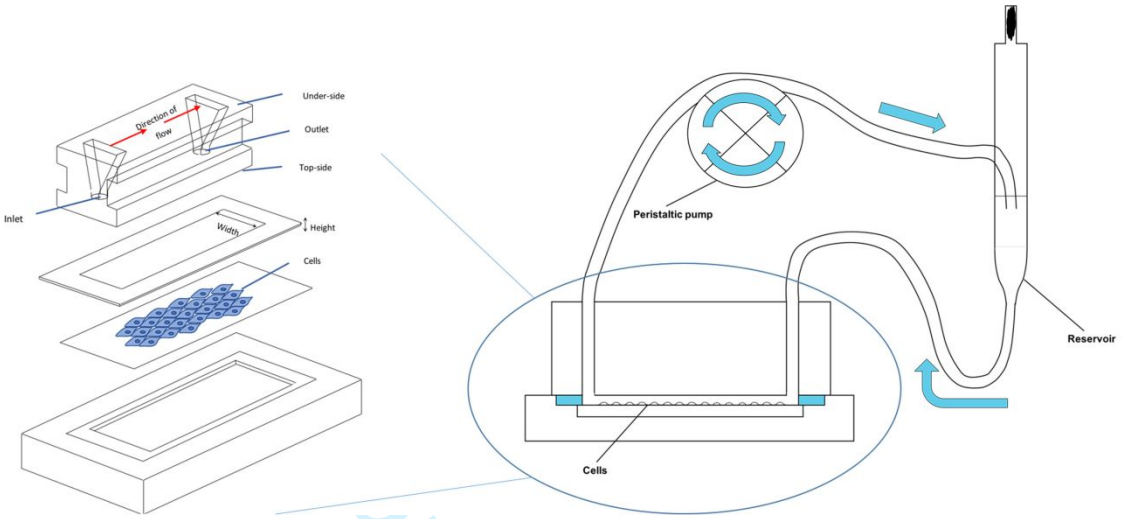
Conflict of interest: No conflicts of interest are declared by authors.

Supplementary figure 1. Schematic representation of the experimental shear stress inducing system in-vitro.



Supplementary figure 2. Representative image of WB of Nrf2 from HUVECs (A) Activation of Nrf2 with different doses of SFN. (B) Expression of mutant type of Nrf2 using Nrf2-Ad-DN. (C) Over expression of Nrf2 using Nrf2-Ad-WT.